TRACHEAL FILLING IN SCIARA LARVAE

MARGARET L. KEISTER AND JOHN B. BUCK

Laboratory of Physical Biology, National Institutes of Health, Bethesda 14, Maryland

Introduction

During insect development the gas-filled tracheae of a given instar become enclosed in larger, liquid-filled, coaxial tubes which are to form the tracheal system of the next instar. At molting the old tracheae are withdrawn and shed with the body cuticle. The new system then fills with gas, either immediately or within a short time, the liquid probably passing through the tracheal wall into the blood or tissues (Weismann, 1863; Palmén, 1877; Keilin, 1924, 1944; Davies, 1927; Wigglesworth, 1938; Keister, 1947, 1948). We shall call the process by which gas replaces the tracheal liquid "tracheal filling."

It is frequently assumed that the gas which appears in the new tracheae of insects with open tracheal systems is atmospheric air which has entered through the spiracles. This assumption is supported by the fact that in some insects the tracheae do not fill with gas unless the spiracles are exposed to free air. In other species, however, the tracheae can fill with gas even when the larva or embryo is submerged in water or surrounded by amniotic fluid, and many aquatic insects with closed systems normally fill their tracheae without apparent contact with an external source of gaseous gas (for literature see Keilin, 1924, and Sikes and Wigglesworth, 1931). It thus appears that in different insects there are at least two distinct mechanisms of tracheal filling, differing as to the source of the gas.

A number of explanations of tracheal filling have been proposed.

Weismann claimed that growth of tracheae (increase in diameter and number) continues after a change in permeability of the lining prevents entry of further liquid. The original liquid then retreats into the finer branches where it is absorbed, and gas enters from the outside or diffuses in from the tissues to occupy the increased tracheal volume. Major objections to this idea are the facts that, at least in Sciara, the tracheae have the same diameter when originally laid down as when they fill, and that no new branches are added near the time of filling (Keister, 1948).

Stadtman-Averfeld (1923) suggested that the violent body movements at molting expel the liquid from the tracheae, after which air enters through the spiracles.

Tillyard (1916), from the equivocal observation that tracheae collapsed when dragonfly larvae were put in KOH, proposed that the gas in the tracheae was CO_2 which diffused in from the hemolymph and displaced the liquid. Keilin (1924) pointed out several difficulties with this hypothesis. An additional objection is that the hydrostatic pressure in the tracheal liquid would have to be less than in the body liquids in order for gas evolution to be confined to the tracheae.

Filling has been attributed to reduction in body hydrostatic pressure at hatching (Davies) or after eclosion (Fraenkel, 1935). However, such reduction would not bring about the disappearance of the tracheal liquid unless the pressure had pre-

viously opposed some other force (e.g., osmotic) tending to withdraw the liquid into the body. Furthermore, it would not necessarily lead to the concurrent appearance of gas in the tracheae except in insects with spiracles open to the air. In closed tracheal systems, reduction in hydrostatic pressure might indeed bring about a preferential evolution of gas in the tracheal liquid (in the unlikely event that the tracheal liquid was higher in dissolved gases than the other body liquids) but would not account for the disappearance of the liquid itself. In any case, the facts that some embryos fill their tracheal systems before hatching, and that many insect eggs apparently have a low internal pressure (Sikes and Wigglesworth), argue against the idea that hydrostatic pressure has any major role in tracheal filling.

From ingenious experiments on the osmotic control of gas movement in the "tracheoles" of mosquito larvae, Wigglesworth (1930) postulated that increased tissue osmotic pressure due to muscular activity at molting could explain tracheal filling. Such a mechanism might conceivably lead to the replacement of tracheal liquid by outside air in insects with open systems, if the tracheal wall were semipermeable and the tracheal liquid markedly hypotonic to the body liquids as Wigglesworth postulated. However, neither of these conditions has been shown directly and unequivocally to occur, and indeed the latter seems rather improbable in view of Keilin's (1924) and Wigglesworth's (1939) opinion that the tracheal liquid is molting fluid, and Keister's demonstration that it originates by direct cytoplasmic transformation.

Wigglesworth later (1938) discovered that mosquito larvae hatched and kept completely under water for some days do not fill during the same stadium, even when returned to contact with air. From this and other considerations he concluded that the essential factor in normal filling was "secretion" of gas by the cells bounding the tracheae (these presumably being inactivated by long hypoxia in the submersion experiments). Gas secretion by hypothetical specialized cells or protoplasm was also invoked by Sadones (1895), Winterstein (1912), von Frankenburg (1915) in connection with this hydrostatic pressure theory, Pause (1918), and Akehurst (1922). A major objection to this proposal is the lack of any direct cytological, physiological and physical evidence of how secretion of gas might occur.

Keilin (1924) suggested that sudden absorption of tracheal liquid by the surrounding tissues (due to "imbibition or to a chemical reaction") would allow gas to diffuse from the tissues into the vacated space, provided the trachea could withstand a pressure of about an atmosphere. Bult (1939) similarly proposed imbibition as the explanation of the movements of tracheolar liquid and gas which Wigglesworth had attributed to osmotic forces. However, in neither Keilin's account nor in Bult's elaborate hypothesis is there any concrete evidence of the existence or nature of imbibitional changes.

An important characteristic of tracheal filling which must be accounted for by any proposed explanation is its great speed. It seems evident that at least the growth hypothesis of Weismann does not meet this requirement, and it has not been demonstrated that any of the other postulated mechanisms do.

The above review indicates that tracheal filling is far from being satisfactorily explained, and that it has not always been recognized that a process which might account for the appearance of gas in the tracheae would not necessarily explain the disappearance of liquid, or vice versa. The present investigation was undertaken in an attempt to clarify some of the problems outlined above.

Material and Methods

Living larvae of the mycetophilid fly *Sciara coprophila* Lintner were used usually at the beginning of the fourth stadium. The anatomy and development of the tracheal system, the process of molting and methods for raising larvae of known age have been described previously (Keister, 1948). Exposure to gases was carried out in brass cylinders each 22 mm. in diameter, 8 mm. high and with a wall 1 mm. thick. Each chamber had lateral inlet and outlet tubes and was cemented by one rim to an ordinary microscope slide. High humidity was maintained by lining the chamber wall with wet filter paper, and by bubbling the incoming gas through water. The larva was placed ventral side up in a minute droplet of water on a coverglass which was then inverted on the greased top rim of the chamber. Larvae so mounted could be observed for many hours, and at all magnifications. The work was done at $25 \pm 3°$ C.

For convenience in finding larvae in the proper stage, cultures were made up in petri dishes. Individuals about to molt are recognized by the fact that they stop locomoting and feeding and lie extended, usually with the anterior end raised, and make only slight and slow movements. Also, the tracheae are less distinct than in younger third instars due to the future fourth instar system enclosing them, and to the fact that usually much of the gas in the third instar system is replaced by liquid shortly before molting occurs. Further characteristic behavior usually involves: a period of axial back-and-forth rotation of the proventricular region of the gut; a period of slow, strong peristaltic waves of the body wall, alternately forward and backward; and finally, repeated bulging of the body just behind the head capsule. Soon thereafter the dorsal wall of the capsule and the cuticle just behind it split longitudinally, and the larva frees its anterior end and crawls forward out of the tubular exuviae, which are anchored to the substratum by viscid strands. Since the new tracheal system normally remains liquid-filled only for a short time after molting, larvae were mounted as quickly as possible after the splitting of the head capsule (usually within 1 minute). It was generally desirable to hasten the larva out of the cast by gentle prodding at the posterior end.

Observations

Normal tracheal filling

As described previously (Keister, 1948), visible gas is not present in the tracheae of newly-hatched (first stadium) Sciara larvae, and is found in only about one larva out of three even at the end of the stadium. When present, however, its extent is very constant. The actual filling process was never observed in spite of very numerous attempts. However, for reasons given in the earlier paper, it is considered unlikely that the gas enters by way of the posterior spiracles, which are the only pair present at this stage of development.

Second and third stadium larvae are less favorable for detailed study than fourth instars because of their smaller size. However, enough younger larvae were studied to show that tracheal filling after the first and second molts is essentially the same as described below.

Following the third molt, gas appears in 3 to 8 minutes and spreads throughout the (fourth instar) system in 1 to 2 minutes. The gas completely fills the principal

trunks almost instantly and apparently at a rather uniform rate. The lateral branches and spiracular connectives do not begin to fill until after the filling of the main trunks is complete, and they fill more slowly. Gas extends to the ends of the finest branches ("tracheoles"). Filling is initiated and progresses without any visible change in the normal locomotion and activity of the larva.

In normal larvae with spiracles in contact with air, careful and repeated observations under high magnification showed that gas first appears at some single point within the principal trunks, usually in one of the first four body segments. Although the suddenness of filling usually made it impossible to be absolutely certain of the exact spot where filling began, in a number of conclusive instances it began at some point other than a spiracle, and in no instance was it seen to begin at a spiracle. Ordinarily the gas spreads progressively and continuously both forward and backward from the starting point, and fills one main longitudinal trunk completely before crossing over by either the anterior or posterior commissures or both to spread through the tracheae of the opposite side. However, under conditions where filling has been interrupted experimentally (see below), it occasionally restarts in a new liquid-filled region, rather than continuing from its original stopping point.

Since the entire new system is still liquid-filled just after molting is completed, the tracheal liquid does not escape with the molted tracheae. Similarly, since the gas can be seen progressing distally into the liquid-filled branches (which end blindly), the liquid does not leave through any of the spiracles. This is particularly convincing after the first or second molt when the new system has only one pair of developed spiracles (anterior), and the gas can be seen passing posteriorly into regions where there are no possible exits. Furthermore, in larvae filling under oil (see below) no escaping aqueous liquid was seen. Also, liquid was sometimes seen to disappear from sctions of tracheae (usually the closed loops of the large lateral trunks) when gas approached simultaneously from both directions as Wigglesworth has also observed. From the above considerations it follows that the liquid passes through the tracheal and tracheolar walls into the blood and tissues as the gas appears.

Tracheal filling in submerged larvae

Since the open tracheal system of Sciara can, and probably normally does, fill independently of the spiracles, it was of interest to ascertain whether or not submerged larvae can fill their tracheae with gas. Of 27 late third instars submerged in a half inch of aerated water, 21 succeeded in molting in the course of 2 days, although they were not able to free themselves completely from the old cuticles for lack of a relatively dry surface for attachment. The tracheae of these larvae became gas-filled except for occasional individual tracheoles or segmental units. Normally no gas passes from the old to the new tracheae; but even if it did under the abnormal circumstances of the test, the amount would be far too small to fill the fourth instar system. As a possible indicator of whether the gas comes from an internal or external source, the experiment was repeated with freshly-molted larvae (i.e., with completely liquid-filled tracheae) taking care to exclude all air-bubbles from the dishes. Twenty or more larvae were used in each of three liquids: ordinary water,

freshly boiled water (the containers being filled to the top and covered), and a 6 mm. layer of mineral oil. In every instance filling began in approximately normal time and proceeded in the usual fashion. These experiments indicate that a visible external gas bubble is not necessary to initiate tracheal filling, in agreement with the previously described observations under high magnification. Furthermore, since the amounts of dissolved gas were probably quite different in the three liquids, the results suggest first that the gas which fills the tracheae of submerged larvae comes from some internal source, and second that filling is independent of O_2 tension over a wide range.

Tracheal filling in gases other than air

To investigate further the nature of the tracheal gas and the role of O_2 in filling, fresh ecdysiasts were exposed to commercial N_2, CO_2 and CO [1] in the gas chambers previously described.

Although there were considerable variations in the individual responses, the larvae were completely immobilized by a few minutes' exposure to any of the gases. In most instances gas appeared in portions of the main tracheae either in normal time or within 30 minutes and usually after the larvae were motionless. However, filling was not completed unless air was admitted to the chamber. Filling could be stopped and restarted repeatedly by alternating exposures to the tank gas and to air.

If the gases used were passed over hot copper gauze before admission to the gas chamber, filling was completely inhibited for an indefinite period (2 hours was the longest exposure tried). If the larva was returned directly to air after a period of complete anoxia not exceeding 75 minutes, filling usually was normal. Longer periods of complete anoxia often led to incomplete or delayed filling, and sometimes to complete and permanent inhibition. Permanently affected larvae died after a few hours, though they might, for a time, resume body movements, gut peristalsis and heartbeat. Filling was not visibly affected by pure medicinal O_2.

The quantitative relations between tracheal filling and O_2 tension, and differences between the specific effects of the individual gases, will be reported in detail in another communication, but for present purposes it may be said that, if given initially, mixtures of 0.3 per cent O_2 and 99.7 per cent CO_2, CO or N_2 suffice to permit gas to appear in the liquid-filled tracheae.

Effect of low temperature on tracheal filling

In freshly-molted larvae placed in small droplets of water on glass kept on melting ice, filling was indefinitely delayed (2¼ hours was the longest exposure tried). When returned to 25°, filling was normal but could be halted and restarted repeatedly by alternately chilling and rewarming the larva. During the exposure to 0° the larvae were practically motionless but responded markedly to mechanical stimulation.

[1] No difference was observed between experiments done in the light and those done in the dark, using a deep red filter to examine the larvae.

Experiments with hydrostatic and osmotic pressure

Attempts were made to prevent filling by exerting pressure from a coverglass on larvae mounted on a slide either in water or in air. It was found impossible to prevent filling from starting even with the greatest pressure which could be applied without bursting the larva.

The larval cuticle of Sciara is somewhat permeable to water, and exposure to hypertonic solutions causes withdrawal of water from the body. Since the consequent lowering of body turgor might accelerate filling, if release of hydrostatic pressure were a factor in filling, freshly molted larvae were immersed in double Ringer's solution. Filling was normal.

Discussion

From the foregoing experiments the following deductions can be made concerning some of the previous explanations of tracheal filling: (1) Tillyard's hypothesis necessitates a gradient between body and tracheal liquids of CO_2, a gas with high aqueous solubility and diffusion coefficient. This would be rather improbable even for larvae in air, but seems quite out of the question in Sciara larvae, in view of their demonstrated ability to fill in 99.7 per cent CO_2. (2) The fact that filling does not occur in larvae completely relaxed by anoxia argues against mechanisms involving a fall in hydrostatic pressure, as do the experiments with coverglass pressure and hypertonic solution. Conversely, the fact that filling can occur in larvae motionless in 99.7 per cent CO_2 runs counter to Wigglesworth's claim that CO_2-narcosis *per se* inhibits filling, and to Standtman-Averfeld's activity theory. Muscular activity as a factor also seems to be ruled out by the lack of filling after the body contractions induced in chilled Sciara larvae by prodding. (3) The inhibition of filling by anoxia argues against both osmotic pressure and imbibition as prime factors in filling, since both are claimed to be enhanced by anoxic catabolism. (4) Weismann and Keilin believed that the tracheoles were the site of absorption of tracheal liquid, but our findings that liquid can leave through trunk walls and that the trunks fill completely before gas enters any of the side branches indicate that the filling mechanism also operates through the main tracheae. This is supported also by the apparently rather uniform rate of filling in the main trunks (where a deceleration would be expected if the liquid were leaving via the side branches). An additional puzzle is that most reports agree that the largest trunks, which have the highest ratio of liquid content to surface area, fill in a matter of seconds; whereas, the fine, thin-walled branches, where it seems that imbibition, osmosis, etc. should be more effective, fill much more slowly. (5) The fact that the tracheal filling of the first instar does not occur for a day or more after hatching (if at all), whereas tissue osmotic pressure might be expected to be highest during the struggles of the embryo at hatching, militates against the osmotic theory. (6) From the fact that the Tenebrio embryo can fill its tracheae either with outside air or with gas derived from tissue fluids, Sikes and Wigglesworth concluded that there is no essential difference between filling in closed and in open systems. Although this conclusion does not appear justified logically, our finding that Sciara, which has an open tracheal system, normally may fill its tracheae with gas from some internal source, suggests that other insects (e.g., Tenebrio) likewise normally may fill as if they had closed systems in spite of opportunity to take in outside gas. (7) Our

demonstrations that (at ordinary temperatures) O_2 is an absolute prerequisite for tracheal filling, and that filling may be delayed by O_2-poor conditions, offer a possible explanation for the observations of Sikes and Wigglesworth that the Lucilia embryo will fill under water, but only if near the surface; of von Frankenberg that Corethra will not fill its tracheae if kept in boiled water; and of Tillyard that dragonfly larvae in O_2-poor water filled slowly. (The first observation was not elaborated by the authors; the second was attributed to insufficient dissolved gas in the surrounding water to serve for filling the vesicles; and the third to "weakening" of the larvae.) (8) The strict O_2-dependence of the initial filling of tracheae makes it questionable whether the "metabolic" movements of tracheolar liquid and gas studied by Bult are brought about by the same mechanism (though both processes are CO-insensitive) since Bult's postulated imbibitional mechanism is apparently anaerobic.

The net result of the above discussion is to eliminate from serious consideration as prime factors in tracheal filling all proposed mechanisms except those postulating "secretion" of gas. As previously stated, however, this concept is so vague that no critical consideration of it is possible at present. Insofar as "secretion" is a metabolic phenomenon, it is compatible with our finding that tracheal filling is absolutely dependent on O_2 and can be indefinitely inhibited by low temperature. However, we have no evidence as to what metabolic change might be involved, except that it is unlikely to be mediated by the cytochrome system. Numerous other aerobic processes (not all necessarily metabolic) are conceivable. Tillyard for example regarded O_2 as essential for a metabolic process by which gases dissolved in the external medium were transferred into the body and liberated in the tracheae.

It might, of course, be assumed that the *initiation* of filling and the actual filling process depend on quite different mechanisms. One could imagine for example that the reaction which requires O_2, and which might be metabolic, simply pulls a trigger which sets off a physical process resulting in tracheal filling. A number of possible mechanisms will be dealt with in another communication, but the evidence already available permits several deductions. Keister (1948) reported that scraps of air-filled third instar tracheae sometimes break off and are left within the new system at molting. The fact that filling will not start, or if previously started will not continue, in such larvae in the absence of oxygen or near 0° C. shows that filling does not progress automatically once gas bubbles are present in the tracheal liquid. The further fact that filling, when it occurs, does not necessarily begin in regions containing tracheal scraps, shows that preformed gas bubbles are not a prerequisite for the initiation of filling. The same conclusions follow from the fact that after filling has been interrupted experimentally it sometimes resumes in a new (liquid-filled) region rather than continuing from its original stopping point. Finally, since filling will occur in gases ranging from tank O_2 to practically pure CO_2, CO or N_2 it is very unlikely that either the initiation or the progress of filling depends on the attainment of any critical ratio between gas concentrations, or upon the presence of any specific gas (except O_2).

Summary

(1) After each molt the new tracheal system of a Sciara larva in air normally remains filled with liquid for 3 to 8 minutes. It then fills spontaneously, rapidly

and completely with gas, beginning at some point in a main trunk. The gas comes from some internal source.

(2) Sciara larvae can molt under aerated water, and can fill their tracheal systems with gas while completely submerged in aerated or boiled water, or mineral oil.

(3) Tracheal filling can occur if the larva is in a mixture of 99.7 per cent CO_2, CO or N_2 with 0.3 per cent O_2, but not in complete absence of O_2.

(4) Tracheal filling is indefinitely inhibited near 0° C.

(5) Filling may be stopped and restarted repeatedly by alternating exposures to anoxic gas and air, or to low and room temperatures.

(6) Initiation and progress of tracheal filling are apparently independent of body movements, body hydrostatic pressure, and critical gas ratios.

(7) It is suggested tentatively that tracheal filling involves a metabolic process.

(8) A review and critique of previously proposed mechanisms of tracheal filling is presented.

LITERATURE CITED

Akehurst, Sydney Charles, 1922. Larva of *Chaoborus crystallinus* (De Geer) (*Corethra plumicornis* F.). *Journ. Roy. Micr. Soc.*, 341–372.

Bult, Tamme, 1939. Over de beweging der vloeistof in de tracheolen der insecten. Proefschrift. Rijks-Universiteit te Groningen.

Davies, W. Maldwyn, 1927. On the tracheal system of Collembola, with special reference to that of *Sminthurus viridis* Lubb. *Quart. J. Micr. Sci.*, **71**: 15–30.

Fraenkel, Gottfried, 1935. Observations and experiments on the blow-fly (*Calliphora erythrocephala*) during the first day after emergence. *Proc. Zool. Soc. Lond.*, 893–904.

Frankenberg, Gerhard von, 1915. Die Schwimmblasen von *Corethra*. *Zool. Jahrb.* (Abt. Allg. Zool. u. Physiol. d. Tiere), **35**: 505–592.

Keilin, D., 1924. On the appearance of gas in the tracheae of insects. *Biol. Rev.*, **1**: 63–70.

Keilin, D., 1944. Respiratory systems and respiratory adaptations in larvae and pupae of Diptera. *Parasitology*, **36**: 1–66.

Keister, Margaret L., 1947. The development and physiology of the tracheal systems of *Sciara*. *Anat. Rec.*, **99**: (4) 46.

Keister, Margaret L., 1948. The morphogenesis of the tracheal system of *Sciara*. *J. Morph.*, **83**: 373–424.

Palmén, J. A., 1877. Zur Morphologie des Tracheensystems. Helsingfors.

Pause, Johannes, 1918. Beiträge zur Biologie und Physiologie der Larve von *Chironomus gregarius*. *Zool. Jahrb.* (Abt. Allg. Zool. u. Physiol.), **36**: 339–452.

Sadones, J., 1896. L'Appareil digestif et respiratoire larvaire des Odonates. *La Cellule*, **11**: 273–324.

Sikes, Enid K., and V. B. Wigglesworth, 1931. The hatching of insects from the egg, and the appearance of air in the tracheal system. *Q. J. Micr. Sci.*, **74**: 165–192.

Stadtman-Averfeld, H., 1925. Beiträge zur Kenntnis der Stechmückenlarven. I. *Culex pipiens*. *Deutsche Ent. Ztg.*, **2**: 105–152.

Tillyard, R. J., 1916. Further observations on the emergence of dragonfly larvae from the egg. *Proc. Linn. Soc. New South Wales*, **41**: 386–416.

Weismann, A., 1863. Die Entwicklung der Diptera im Ei. *Z. wiss. Zool.*, **13**: 159–220.

Wigglesworth, V. B., 1930. A theory of tracheal respiration in insects. *Proc. Roy. Soc. Lond.*, B **106**: 229–250.

Wigglesworth, V. B., 1938. Absorption of fluid from the tracheal system of mosquito larvae at hatching and moulting. *J. Exp. Biol.*, **15**: 248–254.

Wigglesworth, V. B., 1939. The principles of insect physiology. Dutton.

Winterstein, Hans, 1921. Die physikalisch-chemischen Erscheinungen der Atmung. Handbuch der Vergleichenden Physiologie. Fischer, Jena.